Establishment of artemisinin combination therapy as first line treatment for combating malaria: *Artemisia annua* cultivation in India needed for providing sustainable supply chain of artemisinin

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The Plasmodium falciparum malaria is responsible for about 500 million clinical attacks and one million deaths each year, mostly in Africa and southeast Asia. On account of the parasite having become resistant to the conventional antimalarials, artemisinin-based combination therapy (ACT) is the only reliable treatment option. The Roll Back Malaria (RBM) and Millennium Development Goals (MDG) campaigns of WHO and UNDP, respectively, have made little headway, mainly because of paucity of artemisinin in world market. Here we show that the cultivation and processing of Artemisia annua, presently the only resource of artemisinins, in India, in partnerships, can help build a sound supply chain of artemisinin.

Keywords: *Artemisia annua*, artemisinin combination therapy, malarial drug resistance, *Plasmodium falciparum* malaria, supply chain of artemisinin.

MALARIA incidence is on the rise and the clinical disease caused by *Plasmodium falciparum* is widely distributed and entrenched in areas worldwide where climates are suitable for transmission[1,2]. It was estimated[1], by the use of epidemiological, demographic and geographical parameters, that there were 515 million clinical attacks of *P. falciparum* malaria in the year 2002. About 70.9% of the clinical events occurred in Africa, 23.1% in South East Asia, 2.9% in western Pacific region, 2.3% in the East Mediterranean, 0.7% in America and 0.1% in Europe[1,3,4]. The present level of annual global incidence of malaria continues to be about 500 million, 350 million in the African region and 125 million in the South East Asian region, withstanding the global malaria eradication programmes, such as the Roll Back Malaria (RBM)[5,6] initiative which aims to halve the burden of malaria by 2010 and Millennium Development Goal (MDG)[7] that targets to halt the rising incidence of malaria by 2015.

The long-term fight against malaria involves multiple fronts, including vector mosquito control, use of insecticide treated bednets to limit transmission, prompt and accurate diagnosis of malaria afflicted and effective antimalarial therapy for patients. Deaths due to malaria are occurring in increasing numbers because of frequent failure of the conventional treatments using drugs such as chloroquine and sulphadoxine-pyrimethamine, against which *P. falciparum* populations have developed a high degree of resistance[8,9]. Combination therapies (CTs) with formulations containing an artemisinin compound (ACTs) have emerged as a more reliable treatment option[10,11]. The underlying reason for the dependability of artemisinin is the absence of any recorded cases of artemisinin-resistant malaria[12]. World Health Organization (WHO) has recognized the use of ACTs in the treatment of malaria, as a long term measure to control spread of the disease under its RBM programme[13]. For realization of the objectives of RBM and MDC programmes, the world needs ACT treatments for up to 500 million cases of *P. falciparum* malaria that occur annually. The gross insufficiency in supply of ACTs at the present time is related to short supply of artemisinin and its derivatives[7,14].

The amorphane sesquiterpene artemisinin is naturally formed in the shoot of *Artemisia annua* plant[15] (Figure 1). No other organism is known to synthesize artemisinin. Variously prepared leaves of *A. annua* were used as traditional medicine for fever in China for many centuries. A traditional preparation of *A. annua* for the treatment of malaria is now undergoing trials, on the way to development of a standardized remedy[16–18]. Artemether, arteether, arti-

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sunate, artinulate and dihydroartemisinin are some of the antimalarials semisynthesized from artemisinin, which are relatively more potent than artemisinin and are alternatively being used as monotherapies and as component drugs in ACTs, against multi drug-resistant *P. falciparum* malaria[9,19]. Artemisinin and its derivatives are also serving as therapies against schistosomiasis disease caused by the protozoan species *Schistosoma japonicum*, *S. mansoni*, and *S. haematobium*, which cause 1.5 million disabilities each year[20]. Artemisinins also possess lethal activity(ies) against cancer lines, fungi and bacteria[21–24]. *a*-arteether is known to block the function of quinolone-resistant DNA-gyrase in *Escherichia coli*, *Mycobacterium smegmatis* and *M. tuberculosis*[24]. Artemisinin has been shown to be a cancer chemo-prevention agent[25] as well as an immunosuppressant[26] in mammals, and an inhibitor of chloroplast development/photosynthesis and plant growth in higher plants[27]. Apparently, artemisinins possess several kinds of biological activities and the underlying molecular mechanisms are being investigated[28–34]. The disruption of haemoglobin catabolism has been implicated in the parasiticidal action of artemisinin in malaria; the iron catalysis of the endoperoxide bridge of artemisinin generates carbon-centred free radicals, which alkylate heme and parasite proteins, essential for the survival of *Plasmodium*[31–34]. Artemisinin may prove to be active against other protozoan infectious diseases such as leishmaniasis, where the parasite depends on the digestion of haemoglobin to meet its nutritional needs. The absence of artemisinin resistance in malarial parasites may be related to multiple biological activities inherent in the sesquiterpene lactone endoperoxide artemisinin structure (Figure 2), beginning to be revealed by the study of inhibitory actions of artemisinin and its precursors and products on a variety of prokaryotes and eukaryotes.

The artemisinin antimalarials are safely tolerated, have short half-lives and act rapidly in patients, against the young asexual and early sexual forms of *P. falciparum* parasite life cycle[35]. Thus they combine properties of potency, anti-infectivity and protection from resistance development. Loose or fixed-dose combinations of artesunate (AS), artemether (AM) or dihydroartemisinin on the one hand and amodiaquine (AQ), chloroquine (CQ), halofantrine (HF), lumefantrine (LF), mefloquine (MQ), piperaquine

Figure 1. Seeds (left) and leaf (middle) of *Artemisia annua* cv Jeevanraksha (family Asteraceae) plant and artemisinin (right) present richly in the leaves of this cultivar.

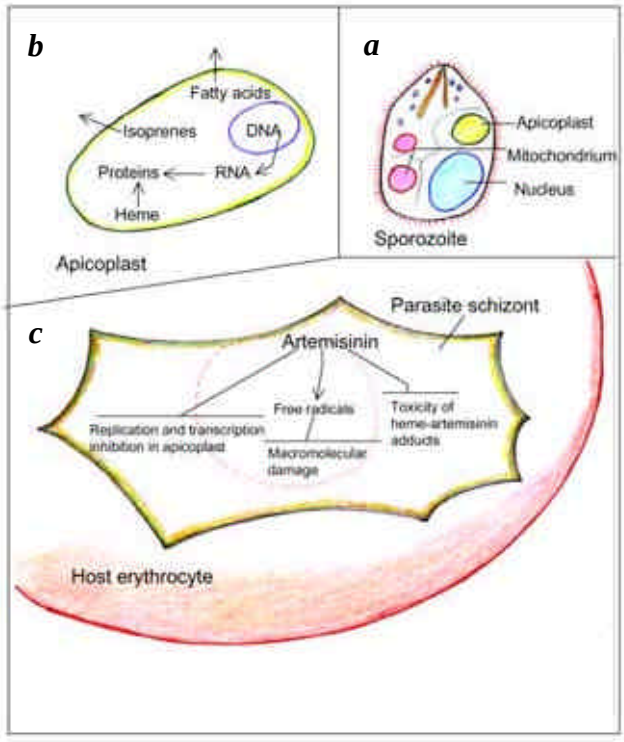

Figure 2. Diagrammatic representation of various kinds of inhibitory actions of artemisinins on the intra-erythrocytic asexual and sexual developmental stages of malarial parasite. ***a***, *Plasmodium falciparum* parasite shown as a sporozoite which is transmitted to man by the bite of an infected mosquito. Sporozoites penetrate the liver cells, multiply asexually, split liver cells and infect red blood cells (erythrocytes). Parasite cells carry apicoplast, a relict plastid (or chloroplast), whose metabolism is essential for the parasite[33]. ***b***, Apicoplast. The apicoplast present in the parasite in all stages of its development is a target of artemisinin. Artemisinin will inhibit the anabolic functions of apicoplast, including those concerned with biosynthesis of heme, isopentenyl diphosphate and fatty acids, essential for the parasite, by way of its inhibition of apicoplastic genome[27,33]. ***c***, Schizont in an erythrocyte. Merozoites liberated by liver cells enter blood corpuscles (erythrocytes) and become trophozoites and schizonts. The parasite multiplies asexually in erythrocytes. Temperature of malaria patient rises when the parasite schizonts growing in erythrocytes burst, releasing merozoites. Merozoites infect new erythrocytes for further asexual multiplication of parasite. Plasmodia also produce gametes in erythrocytes by a sexual cycle of growth. When mosquito sucks blood from a malarial patient, gametes get transferred to it. Fertilization occurs in the gut of the mosquito to serve as initiator of another cycle of malarial infection. Artemisinin has inhibitory action on sexual and asexual intra-erythrocyte stages of plasmodia[28–35]. It stops parasitaemia as well as transmission. The endoperoxide bridge of artemisinin generates carbon-centred, short-lived radicals through its iron-mediated catalysis. These radicals incapacitate a variety of macromolecules, whose activities are essential for plasmodium growth and development. The heme–artemisinin adduct itself is highly toxic[32]. The inactivation of sarco-endoplasmic–reticulum Ca^{2+} ATPase by artemisinin-generated free radicals, further incapacitates asexually and sexually multiplying plasmodia[33].

(PPQ), pyronaridine (PRN), sulphadoxine/pyrimethamine (S/P) or trimethoprim (TMP) on the other hand, have been tried or used/are being used in South East Asia, Latin America and/or Africa[36–45]. The fixed dose combinations available in the form of blister packs are: AS–MQ, Artekin™ and AM–LF, Coartem® (artemeether + lumefantrine or benflumetol) and such fixed dose combinations under development are[9]: AS–AQ, AS–MQ and AS–S/P. Novartis and WHO have entered into an agreement which entails the former to make available Coartem drug to the public sector of the developing countries at a preferential price[9]. In the context of ACTs having been identified as the first line of malarial therapy it is important to remember that widespread or locational foci-specific resistance of *P. falciparum* malaria is recorded against the non-artemisinin drugs that go into ACTs, such as AQ, CQ, HF, MQ, PPQ, TMP and S/P[9,46]; and cross-resistance to LF may also be present on account of similarity of its structure and action to quinine, MQ and HF. It is clear that artemisinin component of ACTs will provide the reliability to ACTs as the first line of antimalarial treatment.

The structure of artemisinin molecule which harbours a peroxide bond in 1,2,4-trioxane heterocycle is highly complex and the total chemical synthesis of artemisinin is uneconomical[47,48]. Recently, a synthetic molecule 1,2,4-trioxolane-7 or OZ277 or RBX-11160, which has in it a pharmocophoric peroxide bond and possesses antimalarial activity has entered the phase-2 and phase-3 trials[49]. It is believed that the molecule will have low production cost, will be easier to formulate for three-day oral treatment regimen, and has good potency and pharmacokinetic properties, although it is structurally much different from artemisinin. The results of clinical trial with OZ277 will be known in about five years from now[14,49]. The structural similarities and differences between artemisinin and OZ277 mean that they may share some biological activities and also possess differential biological activities. Attempts to produce artemisinic acid, an intermediate in the biosynthetic pathway of artemisinin, in genetically modified *Escherichia coli* by transfer of mevalonate pathway genes from yeast and/or *A. annua* and those governing steps subsequent to the compound amorpha-4,11-diene from *A. annua* are in progress. The synthesis of amorphadiene in *E. coli* has been achieved[50,51]. There is deficiency in knowledge about the intermediates and enzymes that catalyse the pathway of artemisinin biosynthesis beyond amorphadiene, to artemisinic acid[52]. It is hoped that *E. coli* strains capable of synthesizing recombinant artemisinic acid will be available in about five years time[14]. It is proposed to derive artemisinin semisynthetically from artemisinic acid produced in genetically engineered *E. coli*. The molecule OZ277 or semisynthetic artemisinin is unlikely to be available in the near future. Thus, the RBM must rely on artemisinin produced from *A. annua* medicinal crop plant.

In view of five years having gone by since the RBM initiative, comprehensive efforts are required immediately to reach ACTs to those who need it worldwide, at affordable costs. Significantly, the progress of RBM programme is limited by the high cost of ACTs[9,14]. While antimalarial drugs such as QC and S/P cost US$ 0.1 to 0.2 per treatment course, the cost of ACT treatment course is US$ 1.20 to 3.50; the cost of AS–AM treatment course[9,12] now is US$ 2.40. Malaria control programmes may make good progress if the cost of ACT course is brought down[14] to US$ 1.0. This means that the cost of production of artemisinin must be cut severalfold by making *A. annua* cultivation and artemisinin extraction processes more cost-effective.

A number of biotechnology projects worldwide are aimed at biopharming/producing by farming a variety of valuable molecules: drugs, vaccines, antibodies, hormones, vitamins, proteins, enzymes, etc. In such programmes, cultivable plants will be serving as hosts to genes of concerned pathway(s) transferred from other organisms. It will be ironic if the *A. annua* plant, whose genome has all the essential genes for artemisinin metabolism, cannot be exploited to produce artemisinin at low costs in desired quantities. This challenging problem is a test case for protagonists of biotechnology and biopharming. Conventional breeding and genetic engineering approaches should allow construction of *A. annua* genotypes rich in artemisinin[53]. Besides, improvements in the cultivation, post-harvest processing, artemisinin extraction and artemisinin storage technologies should permit stable supply of artemisinin at low prices. Indeed, there is need to generate new knowledge and exploit the progress in R&D already made in these directions.

The traditional sources of artemisinin have been the wild and cultivated populations of *A. annua* in certain identified regions of China and Vietnam. To meet the new artemisinin supply challenge, *A. annua* crop cultivation requires to be extended to geographical areas where high yields of artemisinin can be obtained by use of cultivars developed for adaptation to such regions. The often reported[54,55] yield of artemisinin from one hectare (ha) of land in China, Vietnam and Africa is 15 kg. Technologies[56,57] standardized in India allow harvests as big as 75 kg/ha. Their deployment should help bring down the cost of production of artemisinin severalfold.

Considerable progress has been made in the agricultural and chemical technologies of artemisinin production in India[58–60]. Four artemisinin-rich cultivars have been developed, namely Jeevanraksha, Jeevanraksha-2, Arogya and Jwarharti; in whole young leaves of these varieties, the respective artemisinin content is 0.8, 1.0–1.2 and 1.2%. These are poly-cross products of serial selections in the progeny of a cross between accessions with low concentrations of artemisinin. Plants of these varieties complete their life cycle in about one year in the Indo-Gangetic plains. Upon transplantation of 8-week-old seedlings in field in February, second generation seeds are ready for harvest in November[61]. A multi-harvest ratoon cultivation technology has been standardized in which upper herbage is harvested four times to give an yield[56,57] of 75 kg artemis-

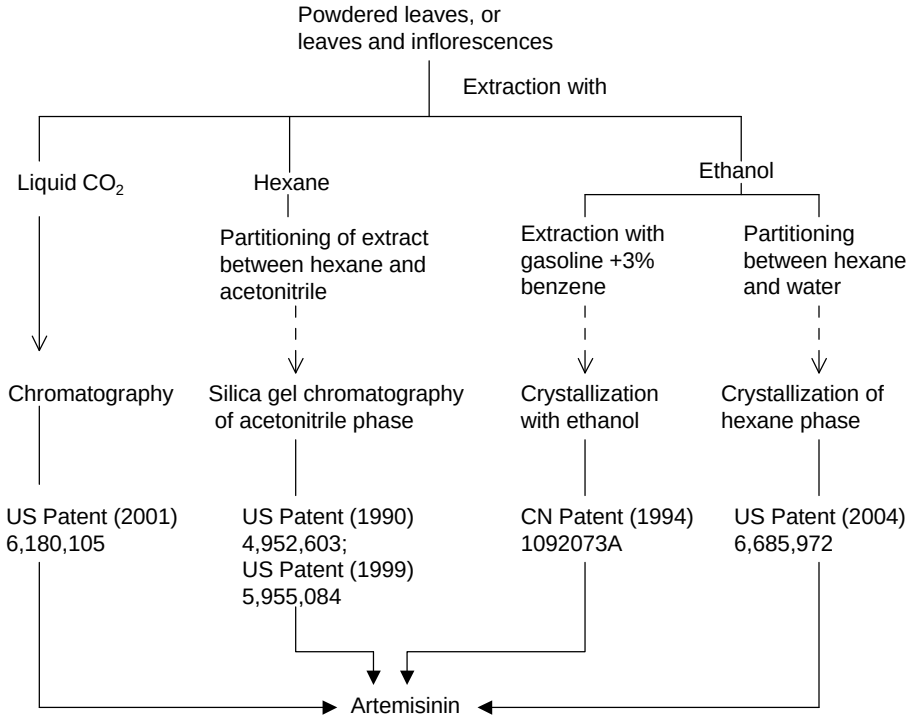

Figure 3. Some processes for extraction of artemisinin from *Artemisia annua* aerial materials[55,60,63,64]. These principally utilize concepts of supercritical fluid and pressurized solvent extraction[64]. A microwave-assisted extraction procedure is also available[65].

inin/ha in contrast to a maximum yield[62,63] of artemisinin at 25 kg/ha reported from Brazil. New processes for the extraction of artemisinin and semisynthesis of artemether, arteether, artesunate and dihydroartemisinin have also been patented[58,64–67]. It is widely realized that combined usage of the most cost-effective agricultural[56,57,63], extraction (Figure 3)[58,64,67–69] and semisynthesis[65,66,70,71] technologies developed in various laboratories worldwide can bring down the cost of artemisinin component of ACT treatment course to less than US$ 1.0.

Indian experience shows that farmers readily adopt industrial crops such as those of essential oil-yielding plants, if they are sure of selling their produce and making a profit[72]. Farmers of the Indo-Gangetic plains who plant menthol mint after winter potato/wheat/Bengal gram crop in March/April, and harvest it before planting rice in July/August, have doubled their profits and India has become the main international supplier of mint oils[72,73]. Farmers will grow *A. annua* if their produce will be picked up by industries/processors at pre-fixed price, yielding a profit of say US$ 100 to 250 per month of crop occupation period. *A. annua* cultivation schedules have also been developed for small farmers, who like to take food crops in their fields, particularly to save a part of the produce for their own use. Such farmers, for example, can follow the following rotations: potato–Artemisia–rice or potato–mint–Artemisia and produce *A. annua* herbage in each hectare of land planted, which is equivalent of about 40 kg artemisinin[57]. All types of *A. annua*-farmers will have as bonus about half a tonne of woody stem and root biomass per kg of artemisinin extracted from their produce[57]. There is need to crop *A. annua* in about 20,000 ha in different seasons and locations, to meet the initial requirements of setting up an international supply chain of artemisinin for the RBM programme.

India should join the global effort in RBM initiative by producing artemisinin in large amounts and thereby make possible the successful implementation of WHO goals. Indian public and private institutions should develop partnerships with their counterparts in other countries who will actively participate in the RBM endeavour. Our analysis of the world scenario further shows that in terms of the continued prevalence of malaria, need and supply of artemisinin for ACT formulations, developments taking place towards availability of artemisinin from genetically modified microorganisms and of synthetic structural analogoue, robustness of artemisinins as safe and stable antimalarials, exploration of artemisinins as a possible cure for certain

cancers and protozoal, fungal and bacterial diseases, Indian S&T institutions should pursue with renewed vigour R&D in *A. annua* and its products. Indeed *A. annua* is a model plant system for biopharming and this plant can be a resource of its own, as well as heterologous products.

Briefly, the RBM programme requires 500 tonnes of artemisinin for use in ACT formulations. Deficiency in the supply chain of artemisinin can be remediated by cultivation of *A. annua* in India. By use of *A. annua*-related agro- and processing-technologies developed in India, the cost of treatment course of ACTs is expected to come down to the desired and affordable level of US$ 1.0. *A. annua* plant can serve as a model for biopharming purposes.

1. Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y. and Hay, S. I., The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*, 2005, **434**, 214–217.
2. Alles, H. K., Mendis, K. N. and Carter, R., Malaria mortality rates in South Asia and in Africa and implications for malaria control. *Parasitol. Today*, 1998, **14**, 369–375.
3. Hay, S. I., Guerra, C. A., Tatem, A. J., Noor, A. M. and Snow, R. W., The global distribution and population at risk of malaria: Past, present and future. *Lancet Infect. Dis*., 2004, **4**, 327–336.
4. Murray, C. J. L. and Lopez, A. D., Mortality by cause for eight regions of the world: Global burden of disease study. *Lancet*, 1997, **349**, 1269–1276.
5. Nabarro, D. N. and Taylor, E. M., The Roll Back Malaria campaign. *Science*, 1998, **280**, 2067–2068.
6. WHO, Antimalarial drug combination therapy. Report of a WHO Technical Consultant, WHO/CDS/RBM/ 2001. 35, WHO, Geneva, Switzerland.
7. Attaran, A. *et al.*, WHO, the global fund, and medical malpractice in malaria treatment. *Lancet*, 2004, **363**, 237–240.
8. Trape, J. F., The public health impact of chloroquine resistance in Africa. *Am. J.Trop. Med. Hyg*. (*Suppl*.), 2001, **64**, 12–17.
9. Olliaro, P. L. and Taylor, W. R. J., Antimalarial compounds: From bench to bedside. *J. Exp. Biol*., 2003, **206**, 3753–3759.
10. White, N. J., Assessment of pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrob. Agents Chemother*., 1997, **41**, 1413–1422.
11. White, N. J., Antimalarial drug resistance and combination chemotherapy. *Philos. Trans. R. Soc. Lond. Ser. B*., 1999, **354**, 739–749.
12. Yeung, S., Pongtavornpinyo, W., Hastings, I. M., Mills, A. J. and White, N. J., Antimalarial drug resistance, artemisinin combination therapy, and the contribution of modeling to elucidating policy choices. *Am. J. Trop. Med. Hyg*. (*Suppl*.), 2004, **71**, 179–186.
13. WHO, Access to antimalarial medicines: Improving the affordability and financing of artemisinin-based combination therapies. WHO document, 2003.
14. Enserink, M., Source of new hope against malaria is in short supply. *Science*, 2005, **307**, 33.
15. Klayman, D. L., Qinghaosu (artemisinin): An antimalarial drug from China. *Science*, 1985, **228**, 1049–1055.
16. Bailey, N. J. C. *et al.*, Prediction of anti-plasmodial activity of *Artemisia annua* extracts: Application of ^{1}H NMR spectroscopy and chemometrics. *J. Pharm. Biomed. Anal.*, 2004, **35**, 117–126.
17. Wilcox, M., Rasoanaivo, P., Sharma, V. P. and Bodekar, G., Comment on 'Randomized controlled trial of a traditional preparation of *Artemisia annua* L. (annual worm wood) in the treatment of malaria'. *Trans. R. Soc. Trop. Med. Hyg*., 2004, **98**, 755–756.
18. Mueller, M. S., Runyambo, N., Wagner, I., Borrman, S., Dietz, K. and Heide, L., Randomized controlled trial of a traditional preparation of *Artemisia annua* L. (annual wormwood) in the treatment of malaria. *Trans. R. Soc. Trop. Med. Hyg*., 2004, **98**, 318–321.
19. White, N. J., Delaying antimalarial drug resistance with combination therapy. *Parasitologia*, 1999, **41**, 301–308.
20. Shuhua, X. *et al.*, Recent investigations of arteether, a novel agent for the prevention of *Schistosomiasis japonica*, *mansoni* and *haematobia*. *Acta Trop*., 2002, **82**, 175–181.
21. Kumar, T. R. S., Khanuja, S. P. S., Jain, D. C., Srivastava, S., Bhattacharya, A. K., Sharma, R. P. and Kumar, S., A simple microbiological assay for the stereospecific differentiation of *a* and *b* isomers of arteether. *Phytother. Res*., 2000, **14**, 644–646.
22. Kumar, S. *et al.*, Method for the use of alpha arteether as an antibacterial and anti-fungal agent. US patent 6,127,405, 2000.
23. Khanuja, S. P. S. *et al.*, Antimicrobial composition and method for producing the same. US patent 6,423,741, 2002.
24. Srivastava, S., Bioprospecting potent plant compounds targeted to inhibit cell wall biosynthesis and DNA replication in *Mycobacterium smegmatis*. Ph D thesis, Devi Ahilya Vishwavidyalaya, Indore, 2002.
25. Lai, H. and Singh, N. P., Oral artemisinin prevents and delays the development of 7,12-dimethylbenz (a)anthracene (DMBA)-induced breast cancer in the rat. *Cancer Lett*., 2005, on-line.
26. Noori, S., Naderi, G.-A., Hassan, Z. M., Habibi, Z., Bathaie, S. Z. and Hashemi, S. M. M., Immunosuppressive activity of a molecule isolated from *Artemisia annua* on DTH responses compared with cycloporin A. *Int. Immunol. Pharm*., 2004, **4**, 1301–1306.
27. Bagchi, G. D., Jain, D. C. and Kumar, S., Arteether, a potent growth inhibitor from *Artemisia annua*. *Phytochemistry*, 1997, **45**, 1131–1134.
28. Jefford, C. W., Why artemisinin and certain synthetic peroxides are potent antimalarials. Implication for the mode of action. *Curr. Med. Chem*., 2001, **8**, 1803–1826.
29. Olliaro, P. L., Haynes, R. K., Meunier, B. and Yuthavong, Y., Possible modes of action of the artemisinin-type compounds. *Trends Parasitol*., 2001, **17**, 122–126.
30. Winstanley, P., Modern chemotherapeutic options for malaria. *Lancet Infect. Dis*., 2001, **1**, 242–250.
31. Meshnick, S. R., Artemisinin: Mechanism of action, resistance and toxicity. *Int. J. Parasitol*., 2002, **32**, 1655–1660.
32. Kannan, R., Sahal, D. and Chauhan, V. S., Heme–artemisinin adducts are crucial mediators of the ability of artemisinin to inhibit heme polymerization. *Chem. Biol*., 2002, **9**, 321–332.
33. Ralph, S. A. *et al.*, Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. *Nature Rev*., 2004, **2**, 203–216.
34. Eckstein-Ludwig, U. *et al.*, Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature*, 2003, **424**, 957–961.
35. Kumar, N. and Zheng, H., Stage-specific gametocytocidal effect *in vitro* of the antimalaria drug qinghaosu on *Plasmodium falciparum*. *Parsitol. Res*., 1990, **76**, 214–218.
36. Nosten, F. *et al.*, Effects of artesunate–mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: A prospective study. *Lancet*, 2000, **356**, 297–302.
37. Ogutu, B. R., Smoak, B. L., Nduati, R. W., Mbori-Ngacha, D. A., Mwathe, F. and Shanks, G. D., The efficacy of pyrimethamine–sulphadoxine (Fansidar) in the treatment of uncomplicated *Plasmodium falciparum* malaria in Kenyan children. *Trans. R. Soc. Trop. Med. Hyg*., 2000, **94**, 83–84.
38. Omari, A. A., Preston, C. and Garner, P., Artemether–lumefantrine for treating uncomplicated falciparum malaria. *Cochrane Database Syst. Rev*., 2003, **2**, CD003125.
39. Ringwald, P., Bickii, J. and Basco, L., Randomized trial of pyronaridine versus chloroquine for acute uncomplicated *falciparum* malaria in Africa. *Lancet*, 1996, **347**, 24–28.
40. van Vugt, M. *et al.*, Artemether–lumafantrine for the treatment of multi drug-resistant *falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg*., 2000, **94**, 545–548.
41. van Vugt, M. *et al.*, Efficacy of six doses of artemether–lumafantrine (benflumetol) in multidrug resistant *Plasmodium falciparum* malaria. *Am. J. Trop. Med. Hyg*., 1999, **60**, 936–942.

42. Denis, M. B. *et al.*, Efficacy and safety of dihydroartemisinin–piperaquine (Artekin) in Cambodian children and adults with uncomplicated *falciparum* malaria. *Clin. Infect. Dis*., 2002, **35**, 1469–1476.
43. Adjuik, M. *et al.*, Amodiaquine–artesunate versus amodiaquine for uncomplicated *Plasmodium falciparum* malaria in African children: A randomized, multicenter trial. *Lancet*, 2002, **359**, 1365–1372.
44. von Seidlein, L. *et al.*, Efficacy of artesunate plus pyramethamine–sulphodoxine for uncomplicated malaria in Gambian children: A double-blind, randomized, controlled trial. *Lancet*, 2000, **355**, 352–357.
45. Marquino, W. *et al*., Efficacy of mefloquine and a mefloquine-artesunate combination therapy for the treatment of uncomplicated *Plasmodium falciparum* malaria in the Amazon basin of Peru. *Am. J. Trop. Med. Hyg*., 2003, **68**, 309–317.
46. Plowe, C., Monitoring antimalarial drug resistance: Making the most of tools at hand. *J. Exp. Biol*., 2003, **206**, 3745–3752.
47. Avery, M. A., Chang, W. K. M. and Jennings-White, C., Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. *J. Am. Chem. Soc*., 1992, **114**, 974–979.
48. Yadav, J. S., Satheesh Babu, R. and Sabitha, G., Stereoselective total synthesis of (+)-artemisinin. *Tetrahedron Lett*., 2003, **44**, 387–389.
49. Vennerstrom, J. L. *et al.*, Identification of an antimalarial synthetic trioxalane drug development candidate. *Nature*, 2004, **430**, 900–904.
50. Picaud, S., Olofsson, L., Brodelius, M. and Brodelius, P., Expression, purification, and characterization of recombinant amorpha-4,11-diene synthase from *Artemisia annua*. *Arch. Biochem. Biophys*., 2005, **436**, 215–226.
51. Martin, V. J. J., Pitera, D. J., Withers, S. T., Newman, J. D. and Keasling, J. D., Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnol*., 2003, **21**, 796–802.
52. Bertea, C. M. *et al.*, Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med*., 2005, **71**, 40–47.
53. Abdin, M. Z., Israr, M., Rehman, R. U. and Jain, S. K., Artemisinin, a novel antimalarial drug: Biochemical and molecular approaches for enhanced production. *Planta Med*., 2003, **69**, 289–293.
54. Ferreira, J. F. S., Simon, J. E. and Janick, J., *Artemisia annua*: Botany, horticulture and pharmacology. *Hortic. Rev*., 1997, **19**, 319–371.
55. Wallart, T. E., Pras, N., Beckman, A. C. and Quax, W. J., Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: Proof of the existence of chemotypes. *Planta Med.*, 2000, **66**, 57–62.
56. Kumar, S. *et al.*, Method for maximization of artemisinin production by the plant *Artemisia annua*. US patent 6,393,763, 2002.
57. Kumar, S. *et al.*, High yields of artemisinin by multi-harvest of *Artemisia annua* crops. *Ind. Crops Prod*., 2004, **19**, 77–90.
58. Jain, D. C. *et al.*, An improved process for the simultaneous production of artemisinin and essential oil from the plant *Artemisia annua*. Indian patent 188,008, 2003.
59. Kumar, S. *et al*., Registration of Jeevanraksha and Suraksha varieties of the antimalarial medicinal plant *Artemisia annua*. *J. Med. Aromat. Plant Sci.*, 1999, **21**, 47–48.
60. Jain, D. C., Bhakuni, R. S., Gupta, M. M., Sharma, R. P., Kahol, A. P., Datta, G. P. and Kumar, S., Domestication of *Artemisia annua* plant and development of new anti-malarial drug arteether in India. *Ind. Res. Prod*., 2000, **59**, 1–11.
61. Gupta, S. K. *et al.*, Morphogenetic variation for artemisinin and volatile oil in *Artemisia annua*. *Ind. Crops Prod*., 2002, **16**, 217–224.
62. De Megalhaes, P. M., Pereira, B., Sartoratto, A., de Oliviera, J. and Debrunner, N., New hybrid lines of anti-malarial species *Artemisia annua* L. In *Proceedings of the Second World Congress on Medicinal and Aromatic Plants* (eds Gibreti, G. *et al.*), *Acta Hortic*., 1999, **502**, 377–381.
63. Delabays, N., Simonnet, X. and Caudin, M., The genetics of artemisinin content in *Artemisia annua* L. and the breeding of high yielding cultivars. *Curr. Med. Chem.*, 2001, **8**, 1795–1801.
64. Kumar, S. *et al.*, Process for isolating artemisinin from *Artemisia annua*. US patent 6,685,972, 2004.
65. Jain, D. C., Bhakuni, R. S., Saxena, S., Kumar, S. and Viswakarma, R. A., Process for the preparation of arteethers from dihydroartemisinin. US patent 6,346,631, 2002.
66. Jain, D. C., Bhakuni, R. S., Sharma, R. P., Kumar, S. and Datta, G. P., Process for preparation of a formulation of dihydro-artemisinin for the control of wide spectrum malaria. US patent 6,362,219, 2002.
67. Jain, D. C. *et al.*, An improved process for the simultaneous production of artemisinin and essential oil from plant *Artemisia annua.* US patent 5,955,084, 1999.
68. Christen, P. and Venthey, J.-L., New trends in extraction, identification and quantification of artemisinin and its derivatives. *Curr. Med. Chem*., 2001, **8**, 1827–1839.
69. Hao, J. Y., Han, W., Huang, S. D., Xue, B. Y. and Deng, X., Microwave-assisted extraction of artemisinin from *Artemisia annua* L. *Sep. Purif. Technol*., 2002, **28**, 191–196.
70. Bhakuni, R. S., Kahol, A. P., Singh, T. and Khanuja, S. P. S., Single pot conversion of artemisinin into artesunic acid. US patent 66,77,463, 2004.
71. Bhakuni, R. S., Singh, T., Kahol, A. P., Tiwari, A., Tandon, S., and Khanuja, S. P. S., Single pot conversion of artemisinin into artemether. US patent 66,83,193, 2004.
72. Kumar, S., Srivastava, R. K., Singh, A. K., Kalra, A., Tomar, V. K. S. and Bansal, R. P., Higher yields and profits from new crop rotations permitting integration of mediculture with agriculture in Indo-Gangetic plains. *Curr. Sci.*, 2001, **80**, 563–566.
73. Singh, A. K., Srivastava, R. K., Kalra, A., Bansal, R. P., Tomar, V. K. S. and Kumar, S., Characteristics of menthol mint *Mentha arvensis* industrial crop production in Indo-Gangetic plains. *Exp. Agric*., 2003, **39**, 199–207.

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